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*Minireview*

# Receptor-mediated calcium entry

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Occupation of membrane receptors can evoke calcium signals by causing depolarisation and activating voltage-operated calcium channels, by triggering internal release, or by stimulating calcium influx processes not gated by membrane potential, receptor-mediated calcium entry, RCME. This brief review considers different possible coupling mechanisms and the proposal that entry can occur from external medium to intracellular store, by-passing the cytosol, and regulated by the state of filling of the store. Recent studies using $Mn^{2+}$ as a probe for RCME are outlined, as are some new electrophysiologic measurements with human platelets and investigations of a novel blocker of RMCE, SK&F 96365.

$Ca^{2+}$; Platelet; $Mn^{2+}$; Fura-2; Endothelium; Patch-clamp

## 1. INTRODUCTION

With the central importance of cell calcium in cell signalling, it is not surprising that diverse mechanisms for $Ca^{2+}$ mobilisation have evolved. We can recognise 3 main classes; $Ca^{2+}$ discharge from internal stores, voltage-gated $Ca^{2+}$ channels and receptor-mediated processes that exploit $Ca^{2+}$ entry from the external medium. An operational definition of receptor-mediated $Ca^{2+}$ entry, RMCE (see [1]) is 'an influx of $Ca^{2+}$ consequent on receptor occupation and not dependent on depolarisation that generates a biologically significant increase in $[Ca^{2+}]_i$'. We thus include significant $Ca^{2+}$ entry through non-specific cation channels as well as entry via $Ca^{2+}$ selective channels.

Various mechanisms for RMCE are shown diagrammatically in Fig. 1. RMCE may occur via mechanisms tightly coupled to the ligand binding site, analogous to the nicotinic receptor/channel complex (1), coupled to receptors via G-proteins (2); these may reasonably be termed receptor-operated calcium channels (ROCC's). More indirect activation could occur via diffusible intracellular second messengers (3). There is also postulated $Ca^{2+}$ entry that is linked to the state of filling of the internal dischargeable $Ca^{2+}$ pool, such that depletion of the pool promotes either entry into the pool by-passing the bulk of the cytosol (4a), or an increased $Ca^{2+}$ permeability of the plasma membrane allowing additional $Ca^2$ influx into the cytosol (4b), see e.g. [1-3]. Physiologically, the $Ca^{2+}$ pool is discharged following receptor occupation and one can therefore consider this indirect type of mechanism under the general heading of RMCE.

We know and understand much less about the function and mechanism of RMCE than about voltage-gated $Ca^{2+}$ entry. Among the reasons for this difference are: (i) the lack of potent specific ligands for RMCE; (ii) the difficulty of reliably detecting and measuring currents associated with RMCE at whole cell or single channel level; (iii) the close temporal and functional association of RMCE with internal $Ca^{2+}$ release; and (iv) the apparent diversity of mechanisms and the possible existence of more than one type of RMCE in many cells.

Possible functions of RMCE include: localised $Ca^{2+}$ signalling, rapid signalling in non-excitable cells, maintenance of $Ca^{2+}$ signals, refilling of discharged internal $Ca^{2+}$ pools, and control $[Ca^{2+}]_i$ spiking, see e.g. [1,4]. It is worth pointing out that in many cells the initial response, and the main source of $[Ca^{2+}]_i$ spikes, is the internal store.

Thus pharmacologic modulation of RMCE could limit pathologic sustained responses while leaving intact the major component of early phase rapid $Ca^{2+}$ signalling.

This paper will note some recent developments in the field, focusing on: studies of RMCE with fluorescent indicator dyes; electrophysiologic data; and some results obtained with novel compounds that can inhibit RMCE in a variety of cells.

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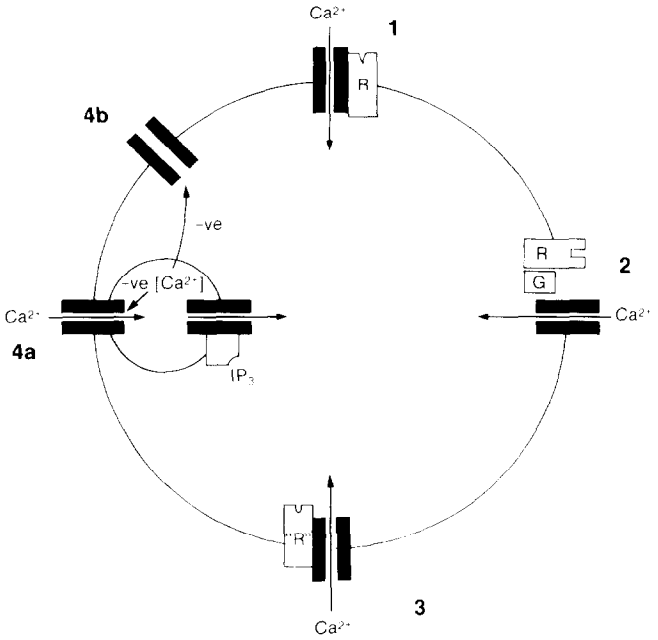


Fig. 1. Various mechanisms of RMCE. 1, receptor-channel complex; 2, receptor coupled to channel via a G-protein; 3, second messenger operated channel; 4a, entry into store regulated by store $Ca^{2+}$; 4b, entry to cytosol regulated by store $Ca^{2+}$.

## 2. STUDIES WITH FLUORESCENT INDICATORS

In many non-excitable cells, agonist-evoked $Ca^{2+}$ signals are often transient in media lacking external $Ca^{2+}$, and are somewhat larger with a maintained plateau (or, if single cells are studied, persistent spiking) in normal external $Ca^{2+}$. The most likely explanation is that the external $Ca^{2+}$ sustains an RMCE. Yet one cannot always exclude a role for external $Ca^{2+}$ in the effective coupling of receptor to $Ca^{2+}$ discharge, and the relative contribution and timing of discharge and entry can be hard to distinguish. $^{45}Ca^{2+}$ influx studies do indicate increased $Ca^{2+}$ entry following stimulation in several cell types; and some recent studies have effectively combined $^{45}Ca$ uptake measurements with monitoring of fura-2 fluorescence [4,5].

We introduced $Mn^{2+}$ as an additional tool for this type of study [6]. Quin2, fura-2 and indo-1 have high affinities for $Mn^{2+}$, and upon binding $Mn^{2+}$ the dye fluorescence is quenched. Agonist-stimulated quenching of the signal from quin2-loaded platelets in the presence of extracellular $Mn^{2+}$ provided the first direct evidence for stimulated influx of extracellular divalent cations into the cytosol of dye-loaded cells [6]. Interpretation of the results using quin2 were complicated because the quench due to $Mn^{2+}$ entering the cytosol was superimposed on the rise in fluorescence due to the agonist-stimulated discharge of internal $Ca^{2+}$ stores. A recent development of this approach using dual wavelength excitation with fura-2 substantially overcomes this problem [7]. One of the excitation wavelengths is chosen to be 340 nm where $Ca^{2+}$ increases the signal, and the other is chosen at the isostilbic wavelength, 360 nm, where the fluorescence of fura-2 is insensitive to $[Ca^{2+}]_i$. $Mn^{2+}$ suppresses the fluorescence at all wavelengths and thus the 360 nm excitation signal provides a monitor for $Mn^{2+}$ influx uncontaminated by any simultaneous changes in $[Ca^{2+}]_i$.

Our group has used the $Mn^{2+}$ approach to extend the original studies with platelets and to examine agonist-evoked $Mn^{2+}$ entry in human neutrophils and endothelial cells [7,8]. In these cells $Mn^{2+}$ appears to serve as an effective surrogate for $Ca^{2+}$ entry, at least through some pathways; interestingly a detailed analysis of thrombin-evoked $Mn^{2+}$ entry in platelets gave a hint of one type of $Ca^{2+}$ entry non-permeant to $Mn^{2+}$ [9]. Also Merritt and Hallam [10] could not detect $Mn^{2+}$ entry into carbachol-stimulated parotid cells under conditions in which RMCE was strongly indicated by other data. An important finding, particularly from the work on endothelial cells, is that $Mn^{2+}$ entry appears to be regulated at least in part by the state of filling of the dischargeable $Ca^{2+}$ stores rather than actual occupancy of receptors.

This has been examined in some detail by brief application of histamine to superfused, single fura-2-loaded human umbilical vein endothelial cells followed by application of $Mn^{2+}$ to assess the rate of $Mn^{2+}$ entry. The initial rate of entry showed a striking inverse relation to the state of fullness of the $Ca^{2+}$ store [11] consistent with mechanism 4a or 4b in Fig. 1.

We have also studied the time-course of agonist-evoked fura-2 signals in several cell types using both standard cuvette fluorescence, and stopped-flow measurements for rapid sub-second kinetics. Internal $Ca^{2+}$ release evoked by maximal agonist concentration occurs with the delay of approx. 60 ms in parotid cells [12], 200–300 ms in platelets [13], and 500–800 ms in neutrophils [14] and endothelial cells (R. Jacob, personal communication). In almost all instances we found that $Ca^{2+}$, or $Mn^{2+}$, entry occurred after or at least no sooner than the evoked internal $Ca^{2+}$ release. These temporal relations are consistent with mechanisms 4a and 4b (store-control of entry) or mechanism 3 if the second messenger mediation of entry is no faster than that for internal release. However, in one instance, with ADP stimulation of human platelets, we find a very early phase of $Ca^{2+}$ or $Mn^{2+}$ entry with a delay of less than 20 ms [9,13] strongly suggesting that mechanism 1 or 2 of Fig. 1 applies to these particular purinergic receptors and the associated $Ca^{2+}$ entry.

## 3. ELECTROPHYSIOLOGIC STUDIES

Perhaps the most widely recognised ROCC's are those detected actually in excitable cells; namely, ATP-activated channels in arterial smooth muscle where both

whole cell $Ca^{2+}$ currents and single channels were recorded [15], and the NMDA type of glutamate receptor channel in central neurones (e.g. [16]). Very recently evidence for receptor-operated channels that are cation permeable and can carry a $Ca^{2+}$ (or $Ba^{2+}$) current have been found by patch-clamp studies of non-excitable cells e.g. in BALB 3T3 cultured cells stimulated by insulin-like growth factor [17], and in our own cell-attached patch-clamp studies of ADP-activated channels in intact human platelets [18]. ADP applied in the pipette, but not via the bath, induces single channels; this finding implies a direct or tight coupling rather than coupling via diffusible second messengers. The channels are permeable to $Na^+$ and $Ba^{2+}$, but not to $Cl^-$, with a conductance of approx. 11 pS at the resting potential. They are blocked by $Ni^{2+}$, as is the ADP-evoked $Ca^{2+}$ or $Mn^{2+}$ entry detected by fura-2. Thus we suspect that ADP acts via ROCC's to evoke its very rapid responses in human platelets.

Evidence is emerging for second messenger activated Ca channels, SMOCCs [19], in a variety of cells and experimental paradigms. Von Tscharner et al. [20] reported data suggesting that a $Ca^{2+}$ permeable channel was activated by elevated $[Ca^{2+}]_i$ in human neutrophils. If this mechanism is present it is not probably the main pathway since one can find experimental conditions where agonists evoke $Ca^{2+}$ or $Mn^{2+}$ entry at basal $[Ca^{2+}]_i$ (e.g. [7]). Inositol trisphosphate has been proposed as an activator of $Ca^{2+}$ entry from work with excised inside-out patches of lymphocyte membranes, and from whole cell recordings of mast cells with inositol trisphosphate applied via the pipette [21,22]. In lacrimal cells studied with internally perfused patch-pipettes the results point to a cooperative action of inositol trisphosphate and inositol tetrakisphosphate in activating $Ca^{2+}$ entry [23]. In none of these studies does the evidence conclusively distinguish between mechanism 3, which one might at first sight assume to be operating, and either mechanism 4a or 4b; these latter mechanisms could apply since the opening of the inositol trisphosphate-activated channel in the internal store could result in an inward current by a conductive pathway across the plasma membrane, either through the store and via the $InsP_3$ channel or across the plasma membrane.

This brief account is by no means comprehensive, but serves to introduce this complex topic and point to some of the proposals for the different routes of RMCE, and different intracellular controls on calcium flux.

## 4. BLOCKING RECEPTOR-MEDIATED CALCIUM ENTRY

Two general classes of agents have been employed to block voltage-dependent $Ca^{2+}$ entry. A diverse range of organic compounds has been identified of which nifedipine, diltiazem and verapamil are leading examples. They were identified from their potent pharmacological actions, typically relaxation of smooth muscle, and subsequently identified as potent and rather specific 'antagonists' of L-type VOC's [24]. Other compounds including certain toxins are now emerging as somewhat specific for other classes of VOC's (e.g. [25]). Also known to block $Ca^{2+}$ currents through VOC's are a range of divalent and trivalent cations including $Ni^{2+}$, $Co^{2+}$, $Cd^{2+}$, and $La^{3+}$ (e.g. [26]). Moreover the apparent affinities of these cations in blocking different classes of VOC's are different so that they may be helpful not only in inhibiting $Ca^{2+}$ currents, but in distinguishing the type of VOC through which the current is passing [25].

These inorganic ions also appear to block RMCE. Different investigators have chosen particular inorganic cations for this purpose, usually $Ni^{2+}$, $Cd^{2+}$ or $La^{3+}$. We have most frequently used $Ni^{2+}$ which has an $IC_{50}$ of about 1.5 mM in inhibiting the $Ca^{2+}$ entry component stimulated by various ligands in neutrophils [7], and a similar apparent potency in human platelets (Leigh and Rink, unpublished results), and human endothelial cells [8]. Interestingly $Ni^{2+}$ also blocks RMCE in parotid cells [9]. $Ni^{2+}$ is equally effective in blocking $Mn^{2+}$ entry as it is with $Ca^{2+}$ entry [6,7]. In human platelets, stimulated by ADP, $Cd^{2+}$ appears to be some 5 times more potent than $Ni^{2+}$ and for that reason may be preferred (Leigh and Rink, unpublished observations). $Co^{2+}$ blocks $Ca^{2+}$ in platelets but also permeates slowly, and is thus less useful than $Ni^{2+}$ which does not measurably permeate.

These inorganic RMCE blockers can be valuable for in vitro experiments, complementing the approach of removing $Ca^{2+}$ from the external medium. For example, $Ca^{2+}$-free solutions are known to have quite diverse effects on membrane function and this may be particularly pronounced when chelators such as EGTA are used to reduce residual $Ca^{2+}$ contamination. In the analysis of sub-second kinetics of $[Ca^{2+}]_i$ signals in human platelets we found it valuable to show that in experiments in which $Ni^{2+}$ blocked $Ca^{2+}$ entry the results were closely similar to those seen with EGTA present; this result suggested that EGTA treatment had not produced artifactual changes in the observed response [13]. However, these inorganic ions are not specific for RMCE and have very limited utility in any in vivo experiments.

At sufficiently high concentrations, usually 1–3 orders of magnitude higher than those required to inhibit voltage-dependent $Ca^{2+}$ entry, the organic '$Ca^{2+}$ antagonists', do reduce, sometimes substantially, RMCE (e.g. [27]). In our hands, the dihydropyridines are considerably more effective than diltiazem in human platelets with $IC_{50}$s in the range 10–20 $\mu$M. There appears to be some selectivity for RMCE in that agonist-evoked internal $Ca^{2+}$ discharge is less affected. Whether these agents are acting at a site similar to that

by which to influence VOCs remains to be determined. It should also be noted that at this concentration these Ca antagonists can have other effects on cellular processes including other types of ion channels, agonist receptors and intracellular $Ca^{2+}$ target proteins. Clearly the absence of specific and potent ligands of RMCE processes is a serious detriment to progress in working out their functional role and the structural basis, and we have looked for new classes of compounds which might act in this way.

One compound we have studied, SK&F 96365, 1-$\beta$-[3-($p$-methoxyphenyl)-propyloxy]-$p$-methoxyphenetyl-1H-imidazole hydrochloride, is able essentially to abolish RMCE, and agonist-evoked $Mn^{2+}$ entry, in a range of non-excitable cells. The $IC_{50}$ is usually around 10 $\mu$M, with little or no effect on agonist-evoked internal release at 30–100 $\mu$M [28]. This compound produces a blockade of RMCE or $Mn^{2+}$ entry evoked by all agonists tested in human platelets, human neutrophils and human umbilical vein endothelial cells. For example, 10 $\mu$M SK&F 96365 greatly reduces the $Mn^{2+}$ influx normally evoked by F-Met-Leu-Phe in fura-2-loaded neutrophils without significantly altering the signal attributable to discharge of internal stores. Interestingly this compound appears to be less effective in blocking $Ca^{2+}$ entry through the ATP-linked channel in smooth muscle cells; 20 $\mu$M SK&F 96365 had no discernible effect in this system. The selectivity that this compound displays for RMCE over internal release does not apparently translate to a selectivity between RMCE and voltage gated $Ca^{2+}$ entry at least in two systems tested. SK&F 96365 inhibited $K^+$-induced $[Ca^{2+}]_i$ elevation in GH3 cells with an $IC_{50}$ in the range 10 $\mu$M and this concentration produced a marked reduction in the $Ba^{2+}$ current attributable to L-type VOCs in patch-clamped smooth muscle cells.

These results encourage us to believe that new classes of compounds may prove to be effective and selective blockers of RMCE, but clearly considerable further progress is needed in identifying more potent and more selective ligands. We have also explored the activity of a related series of glycerol-derived compounds in reducing ADP-evoked $Ca^{2+}$ entry and U46619-evoked aggregation in quin-2-loaded human platelets [29]. The most active compound of this series showed $IC_{50}$ of 1.6 $\mu$M for $Ca^{2+}$ influx; and then was a significant correlation between potency in reducing RMCE and inhibiting aggregation ($r = 0.83$, $P < 0.001$).

## 5. CONCLUSION

These recent studies, and many others, have begun to unravel the complex processes of $Ca^{2+}$ entry and regulation of $[Ca^{2+}]_i$. It is already clear that there will be a large diversity of mechanisms although most cells will share common components. The function of RMCE will likely reflect the end-response of the cell; for example a fast and massive influx of activator $Ca^{2+}$ through the plasma membrane may be the ideal mechanism to achieve rapid response of blood platelets in blood vessels on exposure to ADP. In contrast, it may make a great deal of sense for small refilling fluxes to be triggered during chronic low stimulation of endothelial cells and hepatocytes. It is worth pointing out that the $Ca^{2+}$ flux via RMCE may be very small during prolonged stimulation, 1–3 orders of magnitude less than the (brief) influx obtainable via VOC's in a $Ca^{2+}$ spike.

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